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1. TiTle
Characterization of several phospholipase activities and diacylglycerol/2-monoacylglycerol lipases in rat alveolar macrophages.
AUTHOR: Errasfa, M.
CORPORATE SOURCE: Dep. Prev. Med., Harvard Med. Sch., Massachusetts Gen. Hosp., Boston, MA 02114, USA
SOURCE: BIOCHIM. BIOPHYS. ACTA., (1991) vol. 1085, no. 2, pages 201-8

2. TITLE: Essential role of phospholipase A.sub.2 activity in endothelial cell-induced modification of low density lipoprotein
AUTHOR: Parthasarathy S.; Steinbrecher U.P.; Barnett J.; et al.
CORPORATE SOURCE: Department of Medicine, University of California, San Diego, La Jolla, CA 92093, United States.
SOURCE: Proceedings of the National Academy of Sciences of the United States of America, (1985), 82/9 (3000-3004)

3. Lipolysis of LDL with phospholipase A.sub.2 alters the expression of selected apoB-100 epitopes and the interaction of LDL with cells
AUTHOR: Kleinman Y.; Krul E.S.; Burnes M.; Aronson W.; Pfleger B.; Schonfeld G.
CORPORATE SOURCE: Division of Atherosclerosis and Lipid Research, Department of Medicine, Washington University School of Medicine, St. Louis, MO 63110, United States.
SOURCE: Journal of Lipid Research, (1988), 29/6 (729-743)

4. TITLE: Characterization of several phospholipase activities and diacylglycerol/2-monoacylglycerol lipases in rat alveolar macrophages
AUTHOR: Errasfa M.
CORPORATE SOURCE: Dept. of Preventive Medicine, Harvard Medical School, General Hospital,Boston, MA 02114, United States.

9/0027

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Article title	Characterization of several phospholipase activities and diacylglycerol/2-monoacylglycerol lipases in rat alveolar macrophages
Article identifier	000527609100209C
Authors	Errasfa_M
Journal title	Biochimica et Biophysica Acta - Lipids and Lipid Metabolism
ISSN	0005-2760
Publisher	Elsevier Netherlands
Year of publication	1991
Volume	1085
Issue	2
Supplement	0
Page range	201-208
Number of pages	8
User name	Adonis
Cost centre	
PCC	$22.00
Date and time	Wednesday, May 21, 2003 3:48:35 AM

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Biochimica et Biophysica Acta, 1085 (1991) 201–208

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ADONIS 0005276091002458

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Characterization of several phospholipase activities and diacylglycerol/2-monoacylglycerol lipases in rat alveolar macrophages

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(Received 3 December 1990)

Key words: Phospholipase A_2; Phospholipase C; Diacylglycerol lipase; Monoacylglycerol lipase; Arachidonic acid; (Rat macrophage)

We measured phospholipase activities in both the microsomal and the cytosolic enriched fractions of rat alveolar macrophages by using exogenous arachidonic acid-labeled phospholipids. The microsomal fractions contain a neutral calcium-independent phospholipase A_2 (PLA_2) which acts on substrates phosphatidylcholine (PC) and phosphatidylinositol (PI), a calcium-independent PLA_2 acting on phosphatidylethanolamine (PE), and a neutral calcium-dependent PI-specific PLC. The cytosolic fractions contain calcium-dependent phospholipases: PLA_2 that hydrolyses PC at alkaline pH, and a neutral PI-specific phospholipase C (PLC). The largest release of arachidonic acid from PI occurred with the cytosolic fractions at pH 6 in the presence of calcium. That hydrolysis involved a PLA_2, and a PLC followed by the action of a diacyglycerol and 2-monoacylglycerol lipases. The cytosol also contains a calcium-independent PLA_2 acting on PE. Our investigation shows that rat alveolar macrophages possess a number of phospholipases, as well as diacylglycerol and 2-monoacylglycerol lipases. The above enzymes could play an essential role in the remodeling of membrane phospholipids in resting cells, and the generation of physiologically active lipids in activated cells.

Introduction

Within the cells, it is believed that the hydrolysis of membrane phospholipids provides lipid molecules which are involved in signal transduction and at times in pathophysiological processes. Phospholipase A_2 (PLA_2), PLC and PLD are the key enzymes involved in the hydrolysis of cell phospholipids, resulting in the formation of molecules like eicosanoids [1–4], platelet activating factor [5,6], phosphatidic acid [7,8], inositol 1,4,5-triphosphate and DAG [9].

Several kinds of PLA_2 [10–16], PLC [17–19] and PLD enzymes [20,21] have been characterized and/or partially purified from different cell species. A knowledge of these enzymes in a given cell is required to understand their exact role and involvement in the functions of that cell.

In this study, we attempted to investigate the phospholipases present in rat alveolar macrophages (RAM). Three phospholipid species, nameley, PC, PE, and PI were used as exogenous substrates for cell extracts prepared from RAM.

Here we report the identification and characterization of several phospholipase activities, by their specificity to the substrate and the position of hydrolysis, as well as by their location, pH and calcium dependency. Evidence is presented regarding the release of arachidonic acid from phosphatidylinositol that involves PLC, diacylglycerol lipase and 2-monoacylglycerol lipase activities.

Abbreviations: arachidonic acid, AA; diacylglycerol, DAG; dimethylsulfoxide, DMSO; ethylenglycol-bis(β-aminoethylether)-N,N'-tetraacetic acid, EGTA; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, Hepes; phosphatidylcholine, PC; phosphatidylethanolamine, PE; phosphatidylinositol, PI; phospholipase A_2, PLA_2; phospholipase C, PLC; phospholipase D, PLD; phenylmethylsulfonilfluoride, PMSF; rat alveolar macrophages, RAM.

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Materials and Methods

Distilled, deionized water was used through all experiments. L-3-Phosphatidylcholine, 1-stearoyl-2-[1-^{14}C]arachidonyl (56 mCi/mmol) phosphatidylethanolamine, 1-acyl-2-[1-^{14}C]arachidonyl (56 mCi/mmol), phosphatidylinositol 1-stearoyl-2-[1-^{14}C]arachidonyl (30 mCi/mmol) were purchased from New England Nuclear and Amersham (Boston, MA). Potassium cyanide was purchased from Fisher Scientific. The solvents were of analytical grade. All the other chemicals and kits for enzyme markers were purchased from Sigma Chemical (St. Louis, MO).

Preparation of cells

Male rats (Sprague-Dawley, 375–400 g) were anesthetized with sodium pentobarbital (60 mg per kg of body weight) administered intraperitoneally. The bronchoalveolar cells were collected as described [22], by ten succesive lavages of 8 ml saline at room temperature through a Teflon catheter (2.1 mm × 5.1 cm) tracheal cannula. Cells were pelleted by centrifugation at $350 \times g$ (Beckman GPR centrifuge) for 15 min at 5°C. When red blood cells were present, they were eliminated by exposing the cells to hypotonic solution and centrifuging the cell suspension again. Then cells were resuspended in buffer A: in mM: Hepes 20, NaCl 130, glucose 5.5, KCl 5, $MgCl_2$ 1 (pH 7.4). Cells were counted with an inverted microscope (Olympus) and viability (> 98%) was checked by Trypan blue exclusion. More than 97% of the cells were macrophages as tested by the α-naphthyl acetate esterase assay (Sigma). For technical reasons, cells were used as a cell suspension through all the studies.

Preparation of cytosolic and microsomal fractions

RAM were obtained as described above and kept in buffer A at cold temperature. Cells were then pelleted by 0.3 min centrifugation in Eppendorf centrifuge at $16\,000 \times g$. The cell pellet was resuspended in a 20 mM Hepes (pH 5 and 6) or 50 mM Tris-HCl/Tris-Base buffer (pH 7–8.7). Both buffers contained in mM: KCl 150, $MgCl_2$ 3, EGTA 1, sucrose 340, PMSF 0.02, STI (10 μg/mU and aprotinin (0.17 mg/ml). Cells were then sonicated (0.5 min, % duty cycle 40, out put control 4, Heat Systems-Ultrasonics). The sonicate was centrifuged (0.3 min in Eppendorf centrifuge at $16\,000 \times g$), the resulting pellet PI was descarded. The supernatant was designated as the cell homogenate. The latter was centrifuged for 60 min at $109\,000 \times g$ at 4°C using a TLA-45 fixed angle rotor in the Beckman TL100 Ultracentrifuge. The supernatant representing the soluble enriched cytosolic fraction, called S. The pellet representing the particulate enriched microsomal fraction, called P, was resuspended in the buffer (0.1–0.2 ml) of its corresponding supernatant and then sonicated. The distribution of the following enzyme markers was performed after sonicating the cells in Hepes buffer (pH 7.4): succinic dehydrogenase (SDH) for mitochondria [23] and NADPH-Cytochrome-*c* reductase for microsomes [24] were measured by the method of cytochrome *c* reduction, β-glucuronidase (β-Glu) for lysosomes (modified Sigma assay of [25]) and lactic dehydrogenase (LDH) for cytosol [26]. 5'-Nucleotidase (5'ND) as a marker of plasma membrane was measured by the Fiske and SubbaRow method [27]. Proteins were measured in the samples by the method of Bradford [28].

Assay of phospholipase activity

Phospholipase activity was assayed according to the method described by Ballou et al. [14] with a slight modification [13]. The phospholipid substrate was dried down under nitrogen and resuspended in DMSO. Cell extracts were matched for proteins, and 36 μl of each sample was incubated with 2 μl of the substrate to give 15 μM as a final concentration. 2 μl of calcium chloride or EGTA were added to give a specified final Ca^{2+} and EGTA concentration. Reaction was carried out at 37°C for a given time period, then terminated by adding equal volume of ethanol/2% acetic acid (v/v). A fraction of the reaction mixture was spotted on preheated thin-layer chromatography plate (Whatman, Silica Gel 150), which was developed in the organic phase of the solvent system A: ethyl acetate/trimethylpentane/acetic acid/water; (55:75:8:100, v/v). With this system, phospholipid substrate (R_F 0–0.15), AA (R_F 0.60) and DAG (R_F 0.65) were separated from each other. Solvent system B was also used when specified: chloroform/methanol/ammonium hydroxide 30% (70:35:7, v/v). The radioactivity on the plate was analyzed using a TLC scanner (Berthold, Automatic TLC-Linear Analyser). The result is expressed as a percent of radioactivity associated with each compound.

Statistical analysis

Results were expressed as mean ± S.E. Data were compared using 2-tail paired *t*-test.

Results

Subcellular distribution of enzyme markers

The subcellular fractions of sonicated cells was characterized by the distribution of several enzyme markers. SDH, NADPH-cytochrome-*c* reductase and LDH activities were measured during their linear phase (< 5 min) by mean of a double beam spectrophotometer (Uvikon 860). β-Glu and 5'ND activities were measured after incubating the fractions with the substrate for 1 and 2.5 h, respectively. As shown in Table I, the microsomal and the plasma membrane markers,

TABLE I

Distribution of enzyme markers in cell fractions

Cells ($2 \cdot 10^6$/ml) were resuspended in buffer Hepes (pH 7.4), and sonicated. Then, they were centrifuged in an Eppendorf centrifuge for 0.3 min at $16000 \times g$. The resulting pellet (PI) was resuspended in the same buffer. The supernatant, called homogenate was centrifuged for 1 h at $109000 \times g$ at 4°C, to give a high speed supernatant (HSS), and a pellet (HSP) which was resuspended in buffer Hepes (pH 7.4), then sonicated. 15 to 30 μg of each fraction was used for the assay of succinic dehydrogenase (SDH), NADPH-cytochrome-*c* reductase (NADPH-cyt Red), β-glucuronidase (β-Glu), lactic dehydrogenase (LDH) and 5'-nucleotidase (5'ND). For SDH and NADPH-cyt Red, activity is expressed as nmol of cytochrome *c* reduced per min per mg of protein extract. β-Glu activity is expressed as μg of phenolphtalein produced per hour per mg of protein. LDH activity is expressed as μmol of reduced nicotinamide adenine dinucleotide (NADH) produced per min per mg of protein. 5'ND activity is expressed as μg of phosphorus produced per hour per mg of protein. Data are mean ± S.E. from four to six experiments.

	SDH (nmol/min per mg)	NADPH-cyt Red (nmol/min per mg)	β-Glu (μg/h per mg)	LDH (μmol/min per mg)	5'ND (μg/h per mg)
Pellet P1	10.7 ± 2.8	8.1 ± 3.6	159.3 ± 21.5	80.5 ± 20.1	56.6 ± 16.9
Homogenate	7.5 ± 2.2	5.6 ± 1.7	121.5 ± 21.5	480.6 ± 27.5	74.2 ± 10.6
HSS	1.3 ± 0.7	4.3 ± 2.0	80.8 ± 20.5	610.0 ± 46.9	28.0 ± 7.7
HSP	10.3 ± 1.0	15.4 ± 3.1	105.5 ± 18.6	47.1 ± 6.3	140.8 ± 17.8

NADPH-cytochrome-*c* reductase and 5'ND, respectively, were enriched in the $109000 \times g$ pellet. LDH was highly concentrated in the $109000 \times g$ supernatant. SDH activity in the homogenate was recovered in the $109000 \times g$ pellet. An important amount of β-Glu was found in P1, and less was recovered in the high speed pellet and supernatant. The presence of SDH in the high speed pellet and the distribution of β-Glu are probably due to the disruption of the mitochondrial and lysosomal membranes by sonication.

pH Dependency of phospholipase activity

Soluble (S) and particulate (P) fractions of RAM were prepared in Hepes or Tris buffers as described in the Materials and Method section. 15 to 20 μg of P or S fractions were incubated for 60 min with 15 μM of each radioactive phospholipid in the presence of 2 mM Ca^{2+}. TLC plates were developed always in solvent sytem A, unless specified. With both the P and S fractions, there was some PLA_2 activity to PC and PE which was expressed by the formation of free labeled AA (Fig. 1A, B). In the P fractions, PLA_2 activity to PC showed a high activity at pH 7. In contrast, these fraction hydrolysed PE better at neutral and alkaline pH. In the soluble fractions, there was a PLA_2 activity to PC which prefers the alkaline pH (Fig. 1A). In contrast, the same fractions hydrolysed PE without any specificity to the pH (Fig. 1B). Using solvent system A, labeled AA was the only product generated from PC and PE with the P and S fractions.

The P fractions hydrolysed PI better at pH 7 (Fig. 1 C). At this pH only, the generation of labeled AA was accompanied by the formation of another radioactive compound which has the same R_F (0.65) as standard DAG (1-stearoyl-2-arachidonyl glycerol), and represented about one third the amount of AA. The soluble fractions generated AA from PI with a high activity in the acid pH (Fig. 1C). The soluble fraction prepared at

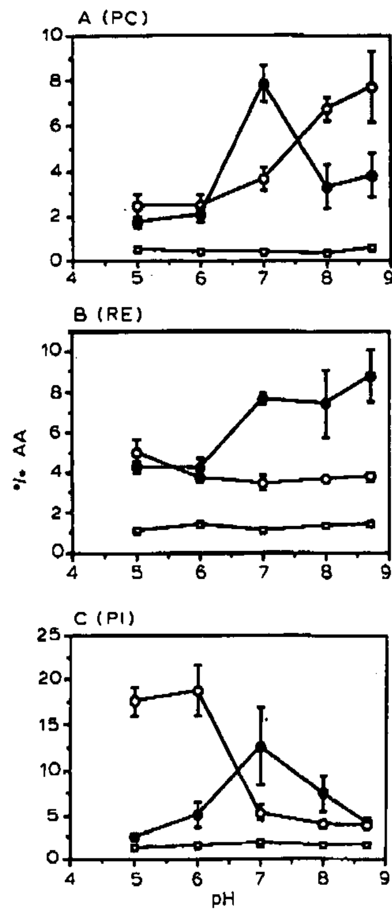

Fig. 1. pH-dependency of phospholipase activity in soluble and particulate fractions. Rat alveolar macrophages were sonicated in Hepes (pH 5 and 6) or Tris buffers (pH 7–8.7). Soluble fraction, S, and the microsomal fraction, P, were prepared by ultracentrifugation as described in Materials and Methods. 15 to 20 μg of each fractions were mixed in a total volume of 40 μl containing 15 μM of the phospholipid substrate, and 2 mM Ca^{2+}. The reaction was terminated after 60 min, and a fraction of each sample was analyzed as described in Materials and Methods. The % of radioactivity associated with AA expresses PLA_2 activity to the added PC (A) and PE (B). In (C), the percent of AA expresses total PI hydrolysis. ○, ● refer to S and P fractions, respectively. □ refer to buffers (B) alone. Data (mean ± S.E.) from four to seven experiments.

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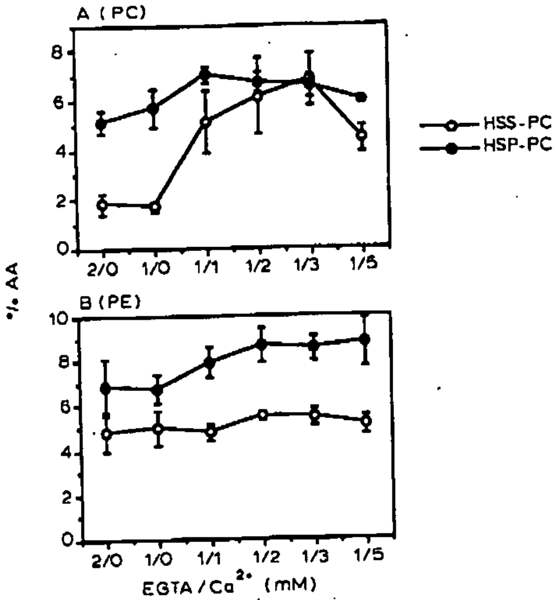

Fig. 2. Effect of calcium on PLA_2 hydrolysis of PC and FE. 15 to 20 μg of the microsomal fraction, P, or the soluble fraction, S, preprared with Tris buffer at pH 7 and 8.7, respectively, were incubated with 15 μM of PC (A) and PE (B) for 60 min. After terminating the reaction, a sample of the reaction mixture was spotted on TLC plate which was devoloped in the solvent system A. Results are expressed as a percent of radioactivity associated with arachidonic acid. ○, ● refer to AA produced by the S and P fractions, respectively. With PC and PE, the basal AA production with the buffers alone (pH 7 and 8.7) is around 0.3% and 0.5%, respectively. Data (mean ± S.E.) from three experiments.

pH 7 induced the generation of AA and an equal amount of DAG.

Effect of calcium on PLA_2 hydrolyses of PC and FE

P7 and S8.7 fractions, prepared at pH 7 and 8.7, respectively, were used to study the effect of Ca^{2+} on their PLA_2 activity to PC and PE. 15 to 20 μg of proteins were incubated for 60 min with 15 μM of each substrate. The hydrolysis of PC in the presence of P7 occurred in the absence of Ca^{2+} (Fig. 2A). When calcium was added, only a small increase occurred in the production of AA. S8.7 exibited an important PLA_2 activity with PC only in the presence of Ca^{2+}.

The hydrolysis of PE by both P7 and S8.7 occurred in the absence of Ca^{2+}, and did not change when Ca^{2+} was added (Fig. 2B).

When TLC plates were developed in solvent system B, which separated very well the three phospholipids (R_F of PI, PC and PE were, 0.35, 0.60 and 0.80, respectively), AA was the only product generated from PC. Even though PE and AA were not separated by solvent B, their corresponding radioactive peak was the only one detected. This finding supports the involvement of a direct PLA_2 hydrolysing activities with PC and PE as substrates.

Effect of calcium on PI hydrolysis

In preliminary experiments, we have found that a PI-derived compound was generated when PI was incubated with soluble fractions which have been prepared in acid buffers. It migrated between standard phosphatidic acid (R_F 0.16, solvent system A) and AA, and less was produced at neutral pH, but none was found at alkaline pH. That compound is referred to as MX (R_F 0.31, M standing for macrophages). Particulate fractions did not induce the formation of MX.

The effect of calcium was studied on the hydrolysis of PI in the presence of soluble fractions prepared at pH 6 and 7, and the particulate fraction prepared at pH 7. 10 μg of each protein extract were incubated with 15 μM PI during 15 min.

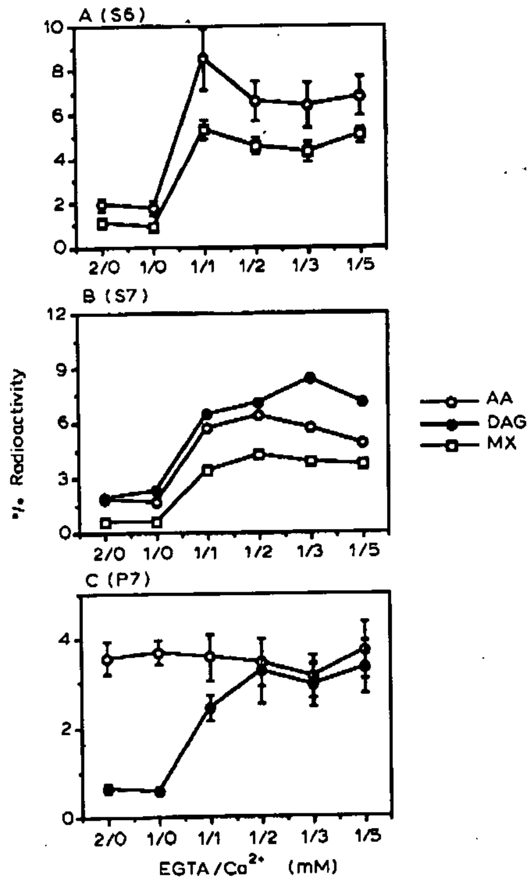

Fig. 3. Effect of calcium on PI hydrolysis. Soluble fractions prepared at pH 6 (S6) and 7 (S7), and particulate fraction prepared at pH 7 (P7) were obtained as decribed in Materials and Methods. 10 μg of each fraction were incubated with 15 μM of PI for 15 min. Ca^{2+} concentration was adjusted to the indicated values. After terminating the reaction, a sample of the reaction mixture was spotted on TLC plate and lipids were analysed as indicated in Materials and Methods. Results are expressed as a percent of radioactivity associated with each compound. ○, ●, refer to AA and DAG, respectively. □ refer to MX compound. The basal production of each compound was around 0.5% for MX and 1% for both AA and DAG. (A): S6, (B): S7, (C): P7. Data (mean ± S.E.) from four to five experiments. For simplicity, S.E. are not given in (B).

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The hydrolysis of PI by soluble fraction S6 was highly dependent on the presence of calcium in the media (Fig. 3A). AA and MX were the main product of PI hydrolysis by S6, even though, a small amount of DAG was sometimes detected. The S7 fraction hydrolysed PI with the generation of DAG, AA and MX. The production of these compounds was calcium dependent (Fig. 3B). From PI, the particulate fraction prepared at pH 7 generated AA in the absence of calcium in the media (Fig. 3C). When calcium was added, the release of AA was not modified, however, hydrolysis of PI increased through the production of newly formed DAG.

Effect of reaction time on PI hydrolysis

When approx. 10 μg of S6 proteins were incubated with PI in the presence of 2 mM Ca^{2+}, there was a rapid decrease of the radioactivity associated with PI, and an increase of AA and MX formation until 15 min (Fig. 4A). Then from 15 to 60 min, labeled AA gained

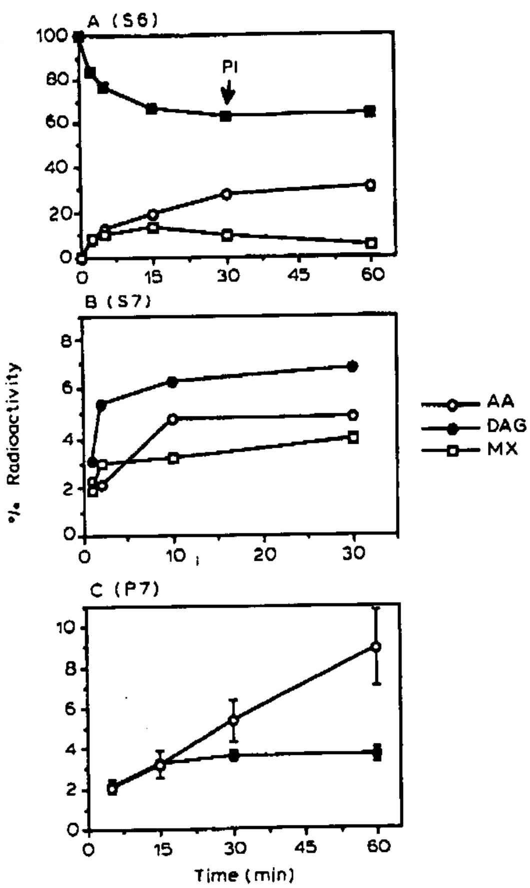

Fig. 4. Time-course of PI hydrolysis. 10 μg of each cell fraction were incubated with 15 μM of PI. Reaction was terminated at the indicated time, and the samples were analysed by TLC. ○, □ refer to AA and MX, respectively. ●, ■ refer to DAG and PI, respectively. (A): S6, (B): S7, (C): P7. Data (mean ± S.E.) from three to five experiments.

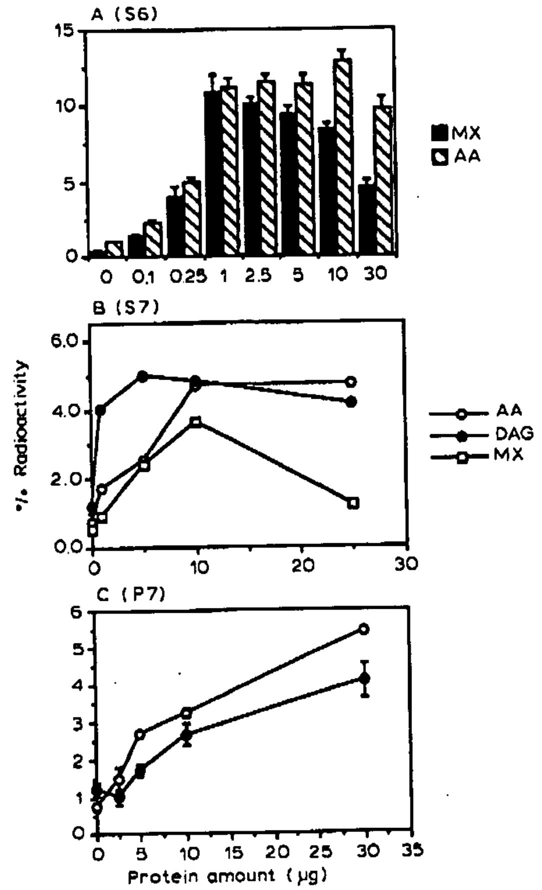

Fig. 5. Effect of protein amount on PI hydrolysis. The indicated amounts of each cell fraction were incubated with 15 μM PI for 15 min. After terminating the reaction, the samples were analysed to quantify each radioactive product. In (A), ▨, ■ refer to AA and MX, respectively. In (B) and (C), ○, □ refer to AA and MX, respectively. ● refer to DAG. (A): S6, (B): S7, (C): P7. Data (mean ± S.E.) from three to four experiments.

11.2 ± 1.8% of the total radioactivity, while MX and PI lost 8.4 ± 0.6% and 2.8 ± 1.8%, respectively.

The hydrolysis of PI by the soluble fraction S7 generated mainly DAG during the first minutes (Fig. 4B). The production of AA, DAG and MX showed a plateau at about 10 min. The production of AA from PI by the particulate fraction P7 was linear through 60 min period, but DAG generation showed a plateau at 15 min (Fig. 4C).

Effect of protein amount on PI hydrolysis

In the presence of the soluble fraction S6, with a 15 min reaction time, the formation of labeled AA and MX started at a protein amount as low as 0.25 μg (Fig. 5A). The formation of labeled AA reached a plateau at 1 μg. Whereas the accumulation of MX was higher at 1 μg, but started to decrease with higher amount of proteins and significantly reduced at 30 μg.

In the presence of low concentration of S7 proteins (1 μg), DAG was the main product of PI hydrolysis

(Fig. 5B). The hydrolysis of PI was higher with 10 μg of S7 proteins. However, with larger amounts of protein, the generation of MX was diminished as occurred with the S6 fraction (Fig. 5A). The hydrolysis of PI by the particulate fraction P7 produced a linear accumulation of both AA and DAG with the indicated amounts of protein (Fig. 5C). When solvent system B was used to analyse the hydrolysis of PI by S6, S7 and P7, no further radioactive compounds were detected other than those separated by solvent system A.

Identification of MX as a 2-monoacylarachidonylglycerol

The migration of MX (R_F 0.31) was found to be the same as that of the following standards; 1-monopalmitoylglycerol, 1-monooleolyglycerol and 2-monopalmitoylglycerol. Therefore, MX as a radioactive product, could be a 2-monoarachidonylglycerol resulting from the hydrolosis at position *sn*-1 of labeled 1,2-diacylglycerol. To check this possibility, we took advantage of a procedure that produce 1-stearoyl,2-arachidonylglycerol from arachidonyl-labeled phosphatidycholine by the action of phospholipace C (*B. Cereus*, Type XI, Sigma). First, PLC (10–12.5 U/ml) was incubated with 40–50 μM of phosphatidylcholine (1-stearoyl-2-[1-^{14}C]arachidonyl) in 0.4 to 0.5 ml of Hepes buffer (pH 7.4) that was used for cell sonication. Reaction was terminated after 1 h incubation time by adding two volumes of methanol, then lipids were extracted according to a modified Folch method [29]. The chloroform phase was evaporated and the residue was resuspended in DMSO and vigorously vortexed. Aliquots (2 μl) of the lipid extract were incubated with the soluble fraction (S6) and Ca^{2+} (3 mM) in a final volume of 40 μl. Reaction was terminated after specified time periods by adding 40 μl of ethanol-2% acetic acid (v/v), then lipids were analysed on TLC using solvent system A as indicated above. This procedure results in the production of DAG representing from 80 to 90% of the total extracted radioactive lipids. When the cytosolic fraction S6 was incubated for 5 min with

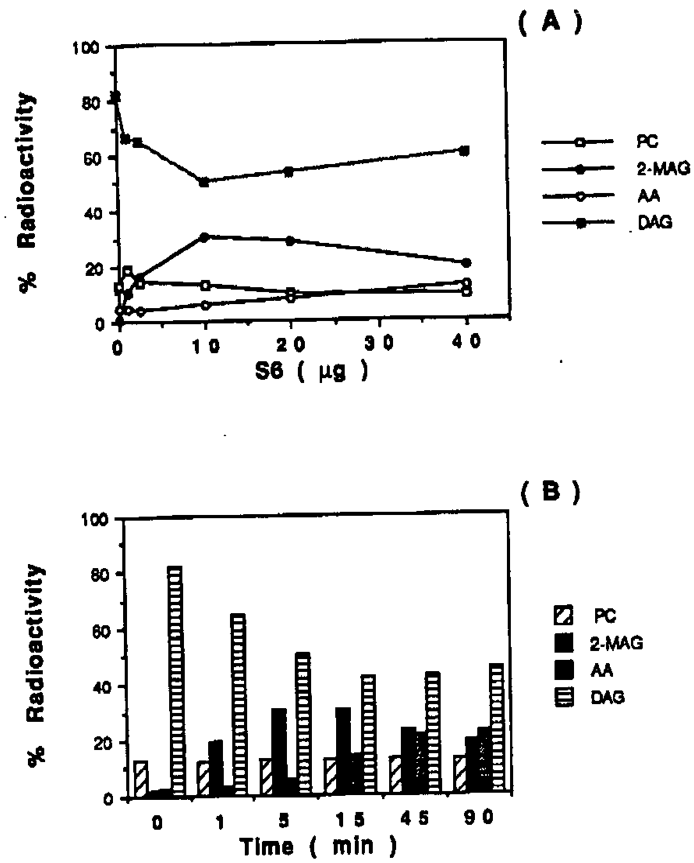

Fig. 6. Generation of 2-monoarachidonylglycerol from 1,stearoyl,2-arachidonylglycerol by S6 fraction. PLC (10–12.5 U/ml) was incubated with 40–50 μM of phosphatidylcholine (1-stearoyl-2-[1-^{14}C]Arachidonyl) in 0.4 to 0.5 ml of Hepes buffer (pH 7.4). Reaction was terminated after 1 h incubation time by adding two volumes of methanol, then lipids were extracted according to a modified Folch method. The chloroform phase was evaporated and the residue was resuspended in DMSO and vigourously vortexed. Aliquots (2 μl) of the lipid extract were incubated with (A) increasing amount of the soluble fraction (S6) for 5 min, or (B) with 10 μg of S6 for the indicated time periods in a final volume of 40 μl containing Ca^{2+} (3 mM). Reaction was terminated by adding 40 μl of ethanol-2% acetic acid (v/v) then lipids were analysed on TLC using solvent system A as indicated in Materials and Methods. In (B), buffer alone has no effect on the amount of the radioactive compounds (data not shown for simplicity of the figure). Data from one representative experiment similar to four others are shown.

DAG resulting from PLC action (Fig. 6A), there was a dose-dependent generation of a radioactive product that has the same R_F (0.31) as MX, therefore, MX is a 2-monoarachidonylglycerol (2-MAG). The hydrolysis of DAG and formation of 2-MAG reached a plateau at 10 μg of protein extract, whereas at 40 μg, MX amount decreased, while arachidonic acid amount increased. With 10 μg of S6 fraction, the generation of 2-MAG from DAG started at one min (Fig. 6B). Alter 15 min, the hydrolysis of DAG reached a plateau, whereas arachidonic acid amount started to increase at the expense of 2-MAG. The low amount of phosphatidylcholine left from the action of PLC was not modified by the above incubations. Buffer alone (Fig. 6B) did not induce any modification in the amount of each of the radioactive compounds (not shown for simplicity of the figure).

Discussion

In this study, we investigated the phospholipase activities present in rat alveolar macrophages. We studied separately the cytosolic and the microsomal enriched fractions by using three exogenous phospholipid substrates. With this method we have been able to characterize several kinds of phospholipases within rat alveolar macrophages.

The microsomal fraction contains a calcium-independent PLA_2 activity to PC and PI with neutral pH optima. With this fraction PI hydrolysis increased upon the addition of calcium through the generation of DAG, while the generation of AA was not modified. Therefore, a PI-specific and calcium-dependent PLC is present in the microsomal fraction. The microsomal fraction also contains a calcium-independent PLA_2 activity to PE which prefers both neutral and alkaline pH.

With the cytosolic fractions, DAG was produced mainly at neutral pH in the presence of calcium. This shows that a calcium-dependent PI-specific PLC is present in the cytosol. It is not known whether the PI-specific PLC which is in the soluble fraction of rat alveolar macrophages is the same enzyme that is found in the particulate fraction. If that is the case, then the difference in PI hydrolysis found between the particulate and the soluble fraction could be due to several factors, such as the presence of other PI hydrolysing enzymes, and the physical environement of PLC.

The hydrolysis of PI was much higher with the soluble fraction prepared at pH 6. With that fraction, and only in the presence of calcium, both AA and 2-MAG (MX) were produced, but less DAG was generated. This suggests on one hand, that a calcium-dependent PI-specific PLA_2 acted on PI, on the other hand, a very active diacyglycerol lipase could be responsible for the formation of 2-MAG. The presence of such an enzyme is supported by the ability of S6 fractions to generate 2-MAG from 1-stearoyl,2-arachidonyl glycerol (Fig. 6).

The low amount of diacylglycerol found upon the incubation of PI with S6 fraction (not shown in Fig. 4A) could be due to its rapid hydrolysis at position *sn*-1 by diacylglycerol lipase at pH 6. In contrast, DAG accumulated in larger amount and less 2-MAG (MX) was generated with cytosolic fractions prepared at pH 7. This result probably means that diacyglycerol lipase is more active at pH below 7.

The 2-MAG (MX) was produced from PI in parallel to AA in the presence of S6 until 15 min (Fig. 4A). Then, while AA production continued to increase, the amount of 2-MAG started to decrease, and only a small decrease occurred in the amount of PI. This modification in the radioactivity associated with each of the three compounds probably means, that the hydrolysis of 2-MAG contributed to the production of AA between 15 and 60 min. When S6 fraction was incubated with 1-stearoyl,2-arachidonyl glycerol that derived from PLC-induced phosphatidylcholine hydrolysis, it induced a similar generation and further hydrolysis of 2-MAG (Fig. 6). These data support the presence of 2-monoacylglycerol lipase in the soluble fraction of macrophages.

A role of diacyglycerol lipase in the release of arachidonic acid was previousely described [30,31]. Later, the release of arachidonic acid as a result of PLC/diacylglycerol lipase/monoacylglycerol lipase activities was also described in several tissues, such as human platelets [32,33], human fetal membrane and decidua vera tissues [34], and in mice peritoneal macrophages [35].

A calcium-dependent PLA_2 that hydrolyses PC with a pH optima at 8.7, and a calcium-independent PE hydrolizing PLA_2 with no pH specificity were also detected in the cytosol. In rat lung cytosol, a calcium-independent PLA_2 acting on PE was previously identified [11].

PI-specific PLC that we describe here in rat alveolar macrophages was also described in other macrophage species [18,36], as well as in human and rat lungs [16], and human platelet cytosol [37]. The properties of this enzyme seem to be different from those of other kinds of PLC found in smooth muscle cells [38] and rat liver lysosomes [17]. The difference between our results and those of Yeats and Bakhle [16], regarding the location of PI-specific PLC could have two explanations, one is that the preparation of PLC by those authors was done from whole rat lung, and second, they homogenized the lung in the presence of 5 mM calcium chloride which may affect the distribution of the enzyme. Indeed, they did not detect any compound related to 2-MAG (MX), probably because their buffer's pH was

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7.4, whereas 2-MAG in our hands was produced mainly at pH 6 with short incubation times.

In summary, rat alveolar macrophages contain several phospholipases, as well as diacylglycerol and 2-monoacyglycerol lipases. The calcium-independent phospholipases seem to be mainly located in the microsomal fraction, whereas the calcium-dependent enzymes were mainly located in the soluble fraction. From this, it seems likely that agonist-induced calcium influx into the cells will activate the cytosolic calcium-dependent phospholipases. Furthermore, calcium-induced translocation of cytosolic PLA_2 to particulate fraction in macrophages [39] and rat brain [40] has been reported.

Acknowledgements

I would like to thank Dr. Alexander Leaf for his valuable discussions. This study was supported in part by National Institutes of Health Grants DK38165,P50 and DK39249.

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